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SNAKE VENOM IMMUNOLOGY: HISTORICAL AND PRACTICAL CONSIDERATIONS

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ABSTRACT

Man has tried to immunize himself against snake venoms and other poisons since the beginnings of history. The scientific study of antivenins began with the work of Henry Sewall in 1887 and has progressed through the present century. Currently, a large number and diversity of monovalent and polyvalent antivenin preparations produced by well-defined protocols are commercially available around the world. These preparations owe much to the pioneering studies of many research workers, but most notably to the work of Sewall, Calmette, Fraser, Brazil, and Noguchi, and more recently to the studies of Boquet and Minton.

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I. INTRODUCTION

Understanding any subject is enhanced by knowledge of its history. The history of snake venom immunology seems no exception. To appreciate it one must view it through those sciences from which it arose -- immunology, bacteriology, virology, allergenic medicine, herpetology, clinical medicine, and toxinology, to note only a few. For that reason, this review will touch upon some of those disciplines in which basic data have contributed in a significant way to our knowledge of snake venom immunology. Obviously, also, some understanding of the chemistry and pharmacology of the snake venoms themselves is essential to venom immunology, as well as a working knowledge of hematology, laboratory medicine, and the skills of treating trauma. It can be seen that snake venom immunology is hardly an entity unto itself but has arisen through multiple biomedical disciplines during the hundred or so years of its course.

Few subjects have stimulated the minds and imagination of man more than the study of snakes and snake venoms. No animal has been more worshipped yet more cast out, more loved yet more despised, more envied yet more caged, and more collected yet more trampled upon than the snake. The essence of the fascination and fear of snakes has lain in their venom. In times past the consequences of bites by venomous snakes were often attributed to forces beyond nature, sometimes to vengeful deities thought to be

embodied in the serpents. To early peoples the effects of snakebites were so surprising and varied, and so violent and sometimes incapacitating, that the snakes and their venoms became shrouded with myth and superstition (1).

II. HISTORY

"It seems," wrote Galen, "that there is nothing more dangerous in life than poisons, and [the bites] of noxious animals" (2). Galen goes on to say that man can avoid these dangers by fleeing them, but as that is not always possible, he may "fall victim to their bite without warning." Like many early Roman and Greek writers, the description of the bites and stings of venomous animals generally portrayed a picture of desperation and frustration. This, in turn, gave rise to a plethora of therapeutic remedies. The origins of most of these early remedies are still obscure. Even the Peri Therion (On Poisonous Animals) by Apollodorus (3), which probably represents the first Greek treatise on poisons, includes many items that appear to be of Near East origins and then passed down through Aristotle, Diocles, Athenaeus, Pliny, Galen, and Sostratus. Of course, the great collection of these remedies for poisoning comes to us through the laborious poems of Nicander in the Therica et Alexipharmaca (4).

The first cures for snake venom poisioning appear to be established in exorcism, a mixture of incantations, chants, laying on of hands, massage, sprinklings, and anointments with various waters, plants, and earths. These were often associated with an elaborate ceremony by a local doctor-priest and appear to have been equally successful or unsuccessful. The practice of exorcism in snake venom poisoning, however, has not been limited by the passing of time. Even today, some form of witchcraft therapy for snakebite is practiced in almost all parts of the world.

The Bibliography of Snake Venoms and Venomous Snakes lists more than 300 "cures" for snakebite that had been suggested up until its publication in 1964 (5). Many of these antidotes are rooted in folklore. Folk medicine, like folklore, transcends both science and education, and tends to derive its remedies from the simple people of each culture, people who in times past lived closest to nature and, supposedly, to nature's secrets. Often they were poorly educated, if at all, and tended to distrust things they did not understand. Such people seek refuge in those things they assume to be inherent in nature, which possibly appear "instinctive" to them. They may create a remedy from an inaccurate observation or a misinterpretation. They may exaggerate an experience, or even a myth, which when repeated to another becomes a "fact". One need only to examine the current scientific literature to see how a falsehood can be promulgated on the unknowing by presumably knowledgeable scientists or physicians. The myth that Antivenin Crotalidae Polyvalent (Wyeth) does not protect against the local tissue effects of crotalid venoms is a good example in this respect.

A. Early Attempts at Immunization

It is difficult to know when man first attempted to experiment with immunizing himself or others. Perhaps Attabus II of Pergamon (c. 170-113 B.C.) and Mithridates VI (120-63 B.C.) of Pontus were the first of the great Greek physicians to dabble with poisons and immunology (6,7). A reader of Nicander, Mithridates acquired a considerable reputation for his readiness and skill in administering poisons as therapeutic agents for a wide variety of diseases. This interest, and perhaps fear in his own art, led him to attempt to concoct a universal antidote, a concoction called Mithridatum that appears to have been employed for almost 1,600 years, or at least through Tudor times (8).

Mithridates is probably equally well-known for his attempt to "immunize" himself by drinking the blood of ducks, which he had been keeping on a ration of one poison or another. Thus, he might be credited with being the first physician to attempt immunization.

It is obvious that the ancients had some idea of the relationship between a disease state and protection against that disease, and it may be that they had a deeper knowledge of this than is generally believed. It is for sure they had observed that individuals who survived a crippling contagious disease seldom contracted the disease again. Those persons whose unpleasant task it was to gather and bury the dead during plagues were often previous victims of that particular disease. Thucydides (390-460? A.D.) (9) notes that while the plague was raging in Athens there would have been no care for the sick and dying if it had not been for those who had recovered from a previous siege of the disease and who served as attendants. Perhaps immunology grew out of these early observations of the Greeks, for we find similar practices during the plagues of the 12th, 13th, and 14th centuries.

Harvey has stated, "Both the Indians and Egyptians immunized themselves by allowing young snakes with a small supply of venom to bite them. Older snakes were used later until full immunity was achieved" (10). I regret that I have not been able to verify this statement, although it certainly is conceivable. Similar statements have been made by a number of writers (see reference 5). According to A. H. Mohammed (personal correspondence, 1978), early Egyptian priests were sometimes bitten by the horned viper (Cerastes cerastes) used in religious rites, but there is no evidence that this was done for immunization purposes. It is not known if these snakes were defanged or milked before being handled, as more recent North American Indian shamans have done (1).

The "Curados de Culebras" Indians of Mexico were said to gain immunity by inoculating themselves with the ground-up teeth of rattlesnakes. Natives of Guiana, Orinoco, the Amazon, and Central Africa, at least by the 19th century, were known to inoculate themselves with snake venoms for immunity (11). It is thought that the reason charmers, priests and witch doctors, among many other native healers, passed their practices down from generation to generation was their belief that by immunizing themselves the immunity could be transferred to their offspring. Further reviews of early attempts at immunization against snake venoms will be found elsewhere (1,11,13,14).

B. Use of Blood and Blood Products During Early Times

The use of blood in the treatment of various disease states was known to the ancients. It was also given for all manners of poisonings, including those caused by venomous animals. The first viable account of the use of various animal bloods in therapeutics was that of Paulus Aegineta, who employed several kinds of blood for ecchymosis about the eyes and as an anti-inflammatory agent in trephining (6). Also, Galen noted the use of blood for various types of poisonings, including those provoked by venomous animals (2). There were numerous reports from the 1400's to the 1600's of the use of animal blood for transfusions in Europe (15-20). In spite of many reported early successes with blood transfusions in the treatment of disease, it became apparent with the work of Denis (21) that infusion of animal blood into a human was fraught with danger, and by the end of the 17th century transfusions were held in some disrepute by most physicians (22).

C. Immunology

It is difficult at best to assign a date for the beginning of any medical discoveries. Edward Jenner is usually credited

with founding the science of immunology in view of his studies on "Cowpox", which might be considered a mutant of smallpox (24). This credit is aptly given, for his work was certainly a milestone in the science of vaccination. Jenner's vaccination technique was a refinement of a technique known as variolation, which had been practiced in Asia for centuries (25-30) and introduced into England by Lady Wortley Montague (28). Jenner's studies on the cowpox vaccine (31) established its effectiveness and led to its replacement of variolation around the world, including the United States (32-39). Although significant contributions to immunology were made on the basis of Jenner's studies, the major extension of his work was not attained until the development of the germ theory of disease and Pasteur's demonstration of methods for producing artificial immunity to such diseases as anthrax, chicken cholera, and rabies (40,41).

It remained for Roux and Yersin in 1888 to demonstrate that immunity could be developed against a toxin by the production of a specific neutralizing antitoxin in the blood of the immunized animal, and that immunity could be transferred to another animal, that is, passively transferred (42). Clinically, this was demonstrated with tetanus antitoxin by Von Behring and Kitasoto in 1890 (43). Ehrlich showed that antitoxins could not only be produced against bacteria, but also against a chemical (44). From these and other works the era of serotherapy emerged. It remained to Metchnikoff (45), however, to suggest that phagocytosis by leukocytes constituted the most important factor in immunity, although this theory gained acceptance only gradually.

D. Snake Venom Immunology

At this point in time, snake venom immunology began to emerge. Almost completely overlooked in the development of our knowledge on antitoxins, and in particular antivenins, has been the work of Henry Sewall (46) (Figure 1). Although Brunton,

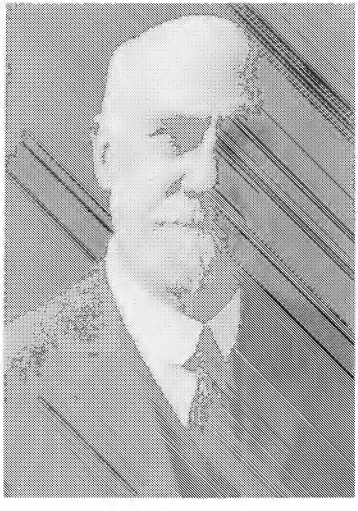

FIG. 1. Henry Sewall (1855-1936). Courtesy Ferdinand Hamburger, Jr. Archives, Johns Hopkins University.

Fayrer, Dumeril, Rudolf, Krehl, and Calmette all subsequently appreciated his important contribution, he did not receive the recognition he so well deserved until years later. When Calmette and a group of French scientists honored Johns Hopkins University with a visit some years after Sewall's experiments, Calmette asked to see the laboratory where the physiologist had carried out his important experiments. Apparently, there were some embarrassing moments. In 1907 Calmette wrote "So long ago as the year 1887, it was shown by Sewall in an important paper on "Rattlesnake Venom" that it is possible to render pigeons gradually more resistant to the action of this venom by injecting them with doses at first very small, and certainly incapable of producing serious effects, and then with stronger and stronger doses . . . he succeeded in making them withstand doses ten times greater than the minimal lethal dose" (47).

The German clinician, Ludolf Krehl, wrote "The foundation for all the works which have been done on animal poisons is to be found in the [work] of Sewall, done at Ann Arbor, Michigan in 1887, the great significance of which was not fully realized until some years afterwards" (48).

In essence, Sewall demonstrated that when pigeons were inoculated with a sub-lethal dose of rattlesnake venom followed by injections of increasing doses to levels above that which would have killed the animals initially, the pigeons developed a resistance to the venom without ill effects. The first page of his famous work is shown in Figure 2.

It can be seen that Sewall initiated his studies with the hope that it might form a worthy contribution to the theory of "Prophylaxis". He notes the analogy between the venom of poisonous snakes and the "ptomaines produced under the influence of bacterial organisms." He developed this analogy from reading the papers by Mitchell and Reichert (49-50) and Wolfenden (51). Sewall had previously observed that various poisons in very small

EXPERIMENTS ON THE PREVENTIVE INOCULATION OF RATTLESNAKE VENOM.

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THE following work was undertaken with the hope that it might form a worthy contribution to the theory of Prophylaxis, and the results obtained during the first stage of its progress are put forward at this time because of the impression that, perhaps, at least their practical significance may induce investigators more fortunately situated for the performance of such experiments to take up the same line of observation. I have assumed an analogy between the venom of the poisonous serpent and the ptomaines produced under the influence of bacterial organisms. Both are the outcome of the activity of living protoplasm although chemically widely distinct, the ptomaines belonging to the group of alkaloids, while the active principles of the venom, according to Mitchell and Reichert[1] and to Wolfenden[2] are of proteid nature.

If immunity from the fatal effects of snake-bite can be secured in an animal by means of repeated inoculation with doses of the poison too small to produce ill effects, we may suspect that the same sort of resistance against germ-disease might follow the inoculation of the appropriate ptomaine, provided that it is through the products of their metabolism that bacteria produce their fatal effects. It is not necessary at this time to consider the bearing of the literature on the subject in question, for there can be drawn from it few, if any, unassailable conclusions.

It is a matter of common experience that with the repeated exhibition of various kinds of poisons in therapeutic doses, more and more of the substance must be employed to produce its physiological action, and, finally, ordinarily fatal doses may be given with impunity. And yet there is reason to believe that this resistance may result from either of two opposite conditions impressed upon the living parts of the body, a pathological or a physiological.

In the first case the sum total energy of the protoplasm is diminished; its irritability is lowered as well as its efficiency as a machine. In the second case the total energy of the protoplasm is not diminished

[1] "Researches upon the Venoms of Poisonous Serpents." *Smithsonian Contrib. to Knowledge*, 674, 1886.

[2] This *Journal*, Vol. VII. p. 327.

FIG. 2. The first page of Henry Sewall's report on immunizing pigeons with rattlesnake venom (Journal of Physiology, 8:203, 1887).

or therapeutic doses eventually lost their effectiveness, and that continually increasing doses had to be used to produce the desired effect. He concluded that these "ordinarily fatal doses may [then] be given with impunity" (46).

Sewall employed the venom of the eastern massasauga, Sistrurus catenatus catenatus, injecting the venom diluted in glycerin along the backs of pigeons just under the skin. It is difficult to determine his initiating or final doses since there is no standard by which they can be measured. Nevertheless, he demonstrated, as in one pigeon, that while the initial lethal dose for a pigeon was two-thirds of a drop of venom, about one month later, having received approximately ten increasing doses of venom, the pigeon could tolerate four and one-half drops without ill effects. Among other matters he noted that: (1) the animal's resistance decreased with time in the absence of sustaining doses of venom; (2) some resistance persisted even after five months; (3) in some experiments he found that if the animals were to die it would be during one of two periods: 3 hours or 15-20 hours after injection; (4) signs of acute toxicity were constant, and consisted of paresis then complete paralysis of the legs, tottering gait, and excessive lacrimal secretions. He also observed that venom kept in glycerin gradually deteriorated. Sewall's report is truly a remarkable contribution. It might also be noted that his work antedates the renowned discovery by Von Behring and Kitasato on diphtheria antitoxin.

In 1889, Kaufmann, using the venom of Vipera berus, obtained somewhat similar results (52), although not with the same high titer as had Sewall, and in 1892, working in Saigon, Calmette began his first experiments on cobra venom, reporting that "It was possible by means of successive inoculations with heated venom to confer . . . a certain degree of resistance to doses invariably lethal to the controls (53).

Kanthack performed a number of experiments on cobra venom and blood, among which was a study on the immunization of animals. From his experiments he concluded that "It is therefore impossible to establish an immunity against the bite of a cobra in this manner [immunization]" (54). This frequently quoted statement has often been used to infer that he questioned the efficacy of an immunization program and a difference with Sewall at this point, but a careful reading of his paper indicated that he was studying some of the problems associated with schedules of immunization, and notes that "with the experience gained by these preliminary experiments, three animals were prepared and accustomed to tolerate large doses . . . they can, however, be accustomed to resist large doses" (controls succumbed to the same doses of venom). There is nothing in this paper that would lead one to question Kanthack's belief in the potential of a well-designed immunization program.

Kanthack also performed another group of experiments in which he mixed the venom of the cobra with fresh cobra blood and injected the mixture into rats, or injected the serum daily. This was followed by a challenging injection of venom. In conclusion he wrote, "This treatment, therefore, holds out no hope for success as it does not even prolong life." Among other things, he demonstrated the importance of dilution as a factor in determining the lethal effect of cobra venom.

By 1894 there were two groups pursuing studies on venoms and antivenins in France. Phisalix and Bertrand, studying the effect of Vipera berus venom on hedgehogs, found them less susceptible to the venom than guinea pigs (55-56), but it must be admitted, as is similarly overlooked in similar experiments today, that merely multiplying the dose of venom on the basis of the body weight of different kinds of animals is not a justifiable accounting for the argument of immunity. It would be interesting to know what Calmette implied in his statement that "the power of

resistance [in one animal as opposed to another] is therefore beyond doubt." I have commented elsewhere on the significance of the variables that need to be considered in differentiating resistance in different animals as modified by bioavailability, passage across membranes, site of action, metabolism and excretion, and as opposed to immunity (57), but such terms as resistance and immunity are still likely to be confused in the literature.

Phisalix and Bertrand demonstrated that guinea pigs or rabbits inoculated with increasing doses of Vipera berus venom developed an immunity (55,56). Calmette also carried out an extensive number of studies on immunization programs and techniques. He came to one conclusion, that animals immunized with cobra venom "are perfectly immune to doses of viper venom or that of other snakes . . . the serum of the vaccinated animals contains antitoxic substances capable of transmitting the immunity to other animals" (57). This concept of a common antitoxin produced by immunization with one venom and viable for the treatment of all snakebites was commonly held by most investigators of the day.

In 1895, Fraser, viewing the possibility that since serpents were immune to their own venom (a rather projected hypothesis), carried out an experiment in which a cat received one-fifth of a minimum subcutaneous lethal dose of a venom orally at two- to five-day intervals on eight occasions, and then increasing doses until the 116th day when the cat received a dose 80 times larger than the original dose without ill effects. The cat was challenged by one and one-half times the minimum lethal dose and had some local edema and skin necrosis, but survived in good health. Further studies were done with white rats. From these experiments he concluded:

> It would therefore appear that although serpents' venom even in enormous quantities fails to produce any toxic effect

> when introduced into the stomach, it still confers upon the animal a certain and not inconsiderable degree of resistance against the toxic effects of subsequent lethal doses of venom. That it does so by causing an antidotal substance to be present in the blood is also manifest from the result of the kitten which had been fed with milk derived from a parent receiving venom by the stomach (58).

However, Calmette was not able to verify these results (47).

These findings were unknown to the present writer in 1958 when Dr. Barry Campbell of Loma Linda University and I carried out an investigation on mice which had been given venom orally from one-half the intravenous LD_{50} to 100 times that amount over a period of 48 days. They were then challenged with an intravenous LD_{50} of the venom, and although the survival rate was higher in the treated than non-treated animals, the differences were not statistically significant for the numbers of animals studied. This experiment might now be repeated using more sensitive detection techniques such as the ELISA.

In the paper mentioned above, Fraser described his experiments on immunizing rabbits and a horse with gradually increasing doses of venom over several months, or until the animals would withstand 30 to 50 times the minimal lethal dose in the rabbits, or 15 times that dose in the horse. He also experimented with various ways of administering his antivenin for testing procedures: (a) mixing venom and antivenin for 30 minutes and then injecting it "nearly subcutaneously", (b) injecting the venom and then the antivenin into different anatomical sites, (c) injecting the antivenin 30 minutes before the venom, and (d) injecting the venom 30 minutes before the antivenin. The best results were obtained with the mixing technique, and from this he concluded that the experiments "appear to also indicate that the antidotism is rather of the nature of a chemical reaction than of a physiological antagonism" (58).

Fraser also made several statements to the fact that there is a remarkable difference between herbivorous and carnivorous animals, with respect to the resistance to snake venom. He concluded that the effect of serpent venom was probably due to its action on the blood and that antivenin should be injected "in the first instance into the part where the venom had been received, before the ligature had been removed . . . and even before the tissues surrounding the wound had been excised" (58). This is a most thoughtful paper.

Calmette continued his studies on the production of antivenin for preventive and therapeutic applications in snake venom poisoning, first using rabbits and guinea pigs. His basic schedule was to accustom the animal to frequent, repeated, gradually increasing doses of the venom (usually cobra venom). Following Fraser's presentation before the Medico-Chirurgical Society of Edinburgh (59), Calmette furthered his interest in producing an antivenin for clinical use and began to inoculate horses and donkeys. Over a period of 16 months some of his animals became tolerant to 80 times the lethal dose of cobra venom. The antivenin had a neutralizing effect of 20,000 units, that is, "one minimal lethal dose per 1,000 g of rabbits by the dose of 0.1 ml of antivenin expressed in a numerical value of 10,000." That is, 1 ml of the serum could neutralize the minimal lethal dose of venom for 10,000 g of rabbit. Actually, Calmette's antivenin had the ability to neutralize 20,000 g of rabbit. This "antivenomous serum" was then prepared for clinical use. The method he suggested was employed by the Institut Pasteur at Lille, and at laboratories in Bombay, Kasauli, Punjab, Philadelphia, São Paulo, and Sidney (47).

Although the therapeutic value of Calmette's antivenin had been noted by Calmette and Fraser, and both agreed that his cobra antivenin was effective against other venoms, Stephens pointed out that the hemolytic principles of the various venoms were not

identical as far as their affinity to Calmette's antivenin was concerned (60). He concluded that antivenins can act only on the venoms employed and those "allied" to it, but not on all snake venoms. Myers found that cobra venom contained two principles, "cobralysin and cobranervin", the former, a hemolytic substance, could be destroyed by heat, while the latter was unaffected. The cobralysin was neutralized by antivenin while the cobranervin was not (61). It soon became apparent from this and other works that the neutralizing capacity of any one antivenin did not cover all snake venoms.

In the United States, McFarland began a series of experiments in 1899 with the cooperation of the H. K. Mulford Company, employing a modification of Calmette's technique for the production of antivenin. Eventually, he prepared antisera for Crotalus, Agkistrodon and Cerastes. He pointed out the difficulties in producing antibodies against the "irritative" principle of venoms, and demonstrated that Calmette's antivenin was not efficacious for Crotalus venom. He reached the same conclusions as Wolfenden, Phisalix, Bernard, and Calmette with respect to antibodies against nerve toxins. He also noted the many variables that could influence the production of an antivenin. His work was published in five papers between 1900 and 1902 (62).

In 1902, Tidswell prepared an antivenin against the venom of Notechis scutatus, but which showed little neutralizing capacity for other Australian venoms (63). The following year Flexner and Noguchi produced several antivenins against Crotalus venom (64-66). Again, the antivenins had no protective action against non-Crotalus species. Lamb produced an effective immune serum in horses against cobra venom. This and his anti-Vipera russelli antivenin were found to be highly specific (67,68). In that same year Noguchi prepared two antivenins in goats, one for Crotalus adamanteus and the other for Agkistrodon piscivorus. Both had specific neutralizing properties (13).

In 1905, Brazil prepared antivenins against Lachesis (= Bothrops) lanceolatus and Crotalus terrificus (C. durissus terrificus), both being specific (69). In 1907, Ishizaka produced an antivenin against Lachesis (= Trimeresurus) flavoviridis using various modified venom solutions. He observed that rectal administration of the venom led to an appearance of antitoxin in the body of the animal, but that the introduction of the venom per os into the alimentary tract failed to do so (70). Kitashima produced an antivenin against the venom of Lachesis flavoviridis in the goat, ox and horse (71). The reader is referred to the fine compendium of Noguchi (13) for a more detailed accounting of these various earlier works.

It would appear that between 1910 and 1920 there was some slowing down in the progress of our knowledge on snake venom immunology. This is understandable, in view of the war, what this author as a child heard referred to as "The Great War", now commonly termed WWI. Interestingly, in combing the literature for our Bibliography (5) on snake venoms some years ago, I found that several of the younger workers in this field, particularly from France and Germany, had lost their lives during that war and that possibly some of their contributions might have been lost. In any event, because of the limitation on space, I will need to skim over the years since 1910. I have reviewed this period in another book (1), but a far more detailed review will be found in the fine works of Phisalix (72) and Pavlovsky (73).

The "modern period" is reflected by the many contributions of a number of workers, the most prominent of which are two of my colleagues, Paul Boquet of France and Sherman A. Minton of the United States. At the risk of calling their works "historic", I wish to indicate just a few of their contributions I consider to reflect much of our current knowledge and thinking on snake venom immunology.

What has been learned from our history of snake venom immunology? Four intrinsic items come to mind:

1. Responses to venom fractions are highly specific, that is, an antibody can differentiate between various forms of an isomeric antigen.
2. The presence of memory for the first experience provides a basis for subsequent responses to that antigen over a shorter time period.
3. Subsequent responses to the venom antigen are greater, or show amplification in both quality and quantity, although a reaction plateau can be reached after repeated challenges.
4. Failure to maintain self-tolerance may result in autoimmune disease.

Although specificity in itself is a feature of many biological systems such as enzyme-substrate reactions, nucleotide interactions and many facets of embrogenesis, the highly specific recognition process within the immunological system for venoms is distinct, as well as important in its phylogeny and ontogeny. Memory, although common to many biological processes, is so unique to venom immunological responses that it becomes an extremely important factor. The quick recognition of a snake venom antigen or antivenin component, as demonstrated by anaphylaxis, indicates how quickly specificity and memory can be brought into effect. Amplification is not a feature unique to snake venom immunological responses. It is seen in quantitative changes in the liver following the administration of many drugs, and elsewhere. In such cases, however, there is no qualitative change in the reactive material as seen with the antibody response, such as the switch from IgM to IgG production. Thus, although specificity, memory, and amplification may be seen in other biological processes, their relationship in the immunological sytem stands apart. In spite of this uniqueness, there are numerous ways in which the response to the immunological process may be expressed in the patient, and therein lies part of the problem in snake venom poisoning. Poisoning due to the venom of

a snake coupled with the patient's immunological sensitivity to a venom protein can sometimes prove to be a very serious therapeutic problem.

E. Complement

While the serum complement system, at least the healing property of the blood, would appear to have been suspected since antiquity, it was not until the discovery of bacteria and methods for culturing them that studies on the bactericidal activity of blood were initiated. In 1884, Grohmann showed that cell-free plasma was capable of destroying bacteria and other microorganisms (84). Based on the work of Metchnikoff (45), a number of investigators demonstrated that organisms injected into the bloodstream were rapidly cleared by phagocytosis in the spleen and other organs. The bactericidal activity of serum was shown to be destroyed by heating, and a number of workers demonstrated additional effects of various temperatures on the bactericidal property of various sera and other body fluids. This property became known as "alexin" (85) or "cytase" (45).

In an inspiring series of experiments, Bordet distinguished between antibody and complement in immune mechanisms (86), and in 1899 Ehrlich and Morgenroth introduced the word "complement" in describing two combining sites, one for red cells and one for complement (87). Subsequent work by Bordet and Gengou showed that there was only one complement but a great many kinds of antibodies (88). Further work on complement was provided by Buchner (85), Ferrata (84), Brand (90) and Gordon et al. (91).

S. Wier Mitchell and colleagues have been credited by many as the scientists who brought attention to initial concepts about complement and snake venom (50), although Fontana should be credited with carrying out the first definitive studies on the effects of snake venom and the blood (91). Mitchell's various works prior to 1900 certainly indicate his concern for blood

changes associated with those produced by the direct action of the venom. He carried on from the observations of Ewing, who showed that when the serum of rabbits was injected with rattlesnake venom, the bacteriocidal property of the serum was lost (93). Stephens and Myers (94) and Stephens (60) showed that cobra venom, incubated with fresh human or animal blood, produced hemolysis, and that this effect was specifically prevented by the antivenin. They also demonstrated that the hemolysis produced by cobra venom could be inhibited by cobra serum. They further showed that the direct hemolytic activity of cobra serum was destroyed by heating at 68°C for 15 minutes, but that this had no effect of the venom hemolytic factor.

It remained for Flexner and Noguchi to put together some of the ideas of complement as they relate to snake venom, and to carry out a number of experiments that clarified the association of the two (66). Using North American crotalid venoms, they observed agglutination of red cells without lysis when venoms in a 0.5% solution were used, but when the same experiment was done with whole blood both agglutination and lysis occurred. When the temperature was restricted to 0°C only agglutination occurred, and at lower concentrations and higher temperatures lysis took place without agglutination. Agglutination activity was destroyed by heating the venoms to 75-80°C for 30 minutes, but the hemolysis-producing property was stable even at 96-100° for 15 minutes. In these and other experiments, Flexner and Noguchi found "that the active principles of the venom require a second substance to manifest their solvent function upon the blood corpuscles," and that this "masks the opening of a new era of study of the haematoxic actions of venoms." They also demonstrated the difference in haemolysins, which they termed "erythrocytolysins" and "leukocytolysins". Their conclusions were as follows:

1. Venom contains principles which are agglutinating and dissolving for leukocytes.

2. The agglutinating principles may be identical for both white and red cells.
3. The dissolving principles for leukocytes are distinct from those for erythrocytes.
4. In order that solution of venomized corpuscles shall occur, a complement-containing fluid is required.
5. The several varieties of white cells of rabbit blood show different susceptibilities to the action of venom (11,13).

Calmette confirmed the observations of Flexner and Noguchi by demonstrating that venom required an additional substance(s) in the blood serum to produce hemolysis, and that this substance was different from the "serum alexines" in that it did not have activating properties at 62°C (47). Keys (95), under the guidance of Ehrlich, and Keys and Sachs (96) confirmed the work of Flexner and Noguchi, and of Calmette, and explained some of the discrepancies in their findings. They found that there were two kinds of blood corpuscles according to their susceptibility to the hemolytic property of the venom. These were (a) the corpuscles that undergo hemolysis by venom in the absence of a second substance, and (b) the corpuscles that became hemolyzed only when "complements" were present. They also demonstrated that even insusceptible kinds of corpuscles could be dissolved by venom if certain suitably fresh sera were introduced. Further, they showed that susceptible corpuscles contained certain substances capable of activating cobra venom. They labeled these "activators" as endocomplements and found them thermolabile.

Further important contributions to blood changes or venom changes in the presence of blood and/or venoms were provided by Noc (97), von Dungern and Coca (98), Morgenroth and Kaya (99), Sachs and Amorokow (100), Ritz (101), Coca (102), Gordon et al. (91), Pillemer et al. (103), Vogt and Schmidt (104), Alper et al. (105), Alper and Balavitch (106), and Alper et al. (109).

According to Alper, almost all recent studies on complement and snake venom have been done with cobra venom. He points out

that while the mechanisms with crotalid venoms may be different from those precipitated by elapid venoms, the end results are similar. The interested reader will find Alper's review on snakes and the complement system an interesting and important work on the development of this subject (108).

At present, complement is considered as having either "classic" or "alternative" pathways. The classic complement system consists of nine numbered protein components, with the first component being divided into three subunits. The components are thus labeled $C1_Q$, $C1_r$, $C1_s$, C2-C9. In reacting with each other sequentially, complement forms products having potent biological effects, including immune adherence, phagocytosis, and cell lysis. The molecular interaction between the first component and IgG or IgM initiates cascading of the classic complement sequence. The alternative or properdin pathway can be activated in the absence of complement binding IgM and IgG by several naturally occurring particulate polysaccharides and lipopolysaccharides, including bacterial endotoxins and IgA aggregates.

The complement system is regulated by inactivators, which are usually enzymes that destroy the primary amino acid sequence of the system, and inhibitors, which do not alter the amino acid chain but rather combine with complement components in such a way as to prevent their further reaction with other components of the system, thus disrupting the cascade.

As Alper has amply put it with respect to snake venoms, "the wheel has now turned full circle with a redirection of scientific interest to the interaction of snake venom and complement after more than half a century of relative dormancy" (108). A renewed interest in cobra venom factor may lead to the structure and precise function of C3, which in turn may yield data on the role of the complement system in allograft rejection and other basic and important biomedical phenomena.

F. Snake Venom Detection

Perhaps one of the earliest techniques for detecting snake venoms in blood or tissues was that proposed by Lamb in 1902. He described a precipitation test for differentiating between "proteids" of different reptile venoms (109). Many years later a modification of the test was employed to detect cobra venom in a fatally envenomated patient (110). In 1957, Minton employed the agar double diffusion method of Ouchterlony to demonstrate the composition of rattlesnake venom. He found that these venoms contained at least four to seven antigenic fractions, three of which appeared commonly shared (79).

In 1967, Russell (111), and also Trethewie and Rawlinson (112), experimented with a simple gel diffusion technique in attempting to detect venom antibodies or antigens. The methods, however, proved to be relatively insensitive and too time-consuming to be of clinical value. The following year, Boche and Russell reported on the use of a passive hemagglutinin test using sheep red blood cells for detecting snake venom in body tissues (113). The test showed accuracy at dilutions greater than 1:200,000 but it was very difficult to perform, as well as being time-consuming. Nevertheless, it was used successfully at the Los Angeles County/University of Southern California Medical Center in a selected group of 25 patients studied over a seven-year period (114). Theakston had difficulty with the method because of controlling conditions for the coupling agents and reagents (115). Indeed, all reagents must be prepared the day of the test, as was done at our Medical Center, if definitive and reproducible results are to be expected.

In 1970 Trethewie again used the Ouchterlony method of gel diffusion in a study on guinea pigs injected with Australian snake venoms. He found considerable overlapping in antigenicity when he used reconstituted dried venoms, but much sharper demarc-

ation when the snake was allowed to bite the guinea pig (116). Ouchterlony techniques were used in determining two deaths following suicides (117,118). Again, the tests were not sufficiently specific to identify the specific snake involved, but they did indicate the probability of snake venom at the family level. Tiru-Chelvam employed immuno-fluorescence techniques for identifying specific sites of localization of snake venoms in vivo. In his summary, he notes that this "study also provides experimental proof of what has been suspected for many years by clinical groups (see reference 119) that the so-called "haemolytic" venoms do indeed have a "neurotoxic" action . . . (120).

In 1974, Greenwood et al. attempted to identify venom in 101 cases of snakebite in Nigeria, employing immunodiffusion and contra-current immunoelectrophoresis. Venom was detected in the wound aspirations of 27 patients, concentrated urine in 19 patients, and blister fluids in 9 patients. In 11 of 26 patients studied, the identity of the offending snake was known, and in these cases the test was positive for that snake (121). In the same year Coulter et al. demonstrated the presence of snake venom in the blood of two patients using a solid phase competitive radioimmunoassay (RIA). The assay demonstrated concentrations of 15 ng per ml in the blood of animals (122). The following year, Sutherland et al. modified the technique for a clinical study in four patients (123). As Coulter et al. subsequently pointed out, the extent of iodination of individual polypeptides can vary considerably (124). The potential for changes at antigenic sites thus makes this test of questionable clinical value (126). This group also noted their experiences with 70 antibody sandwich RIA's in patients bitten by snakes (125).

In their 1978 article (124), Coulter et al. reported the success of their sandwich radioimmunoassay technique, which gave a reliable assay for detecting tiger snake venom in concentrations of 0.1 - 0.4 ng per ml. The authors, however, did not feel

the assay had great clinical value. This concern was also noted by Theakston in 1983, who cited the high equipment cost, requisite technical skill, length of assay time, and shelf-life of the ^{125}I, all of which limited its use to research purposes (115). The RIA, however, demonstrated that labeled antigen-antibody reactions had important laboratory value.

As far back as 1966, Nakane and Pierce (126), and subsequently Massayeff and Maiolini (127) had demonstrated that enzymes might be suitable substitutes for ^{125}I. It remained for Theakston et al. in 1977 to apply the ELISA to the study of snake venoms (128), and in 1978 Pugh and Theakston demonstrated the usefulness of the ELISA in snake venom poisoning in Nigeria (129). Coulter et al. modified the enzyme immunoassay making it possible to obtain determinations in 30-40 minutes (130). Theakston et al. found that the test provided a method of assaying venom potency (131), while Pearn et al. performed ELISA to measure the amount of venom injected into mice following the strike of an Australian common brown snake (132).

Gopalakrishnakone et al. demonstrated the test's usefulness in a study of crude Crotalus durissus terrificus venom and its crotoxin complex (133). Tzeng and Sheik compared two different ELISA enzyme systems to the RIA using cobrotoxin and anti-cobrotoxin. They found the enzyme systems to be as sensitive as the RIA, although the authors felt that the sensitivity with the enzymes was less than expected (134). Since cobrotoxin has only three antigenic determinants, one of which is bound with the solid phase, perhaps with a crude venom the sensitivity would have been greater if there would have been more determinants to bind with the enzyme conjugate.

In 1982 Lwin and Myint found that the ELISA might be a useful tool for assessing the amount of antivenin to be injected by measuring the amount of uncomplexed venom remaining in the blood. This was determined using optical density. They also found that